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Yanina F. Briñoccoli, Sergio Bogan, Dahiana Arcila, Juan José Rosso, Ezequiel Mabragaña, Sergio Delpiani, Juan Diaz de Astarloa, Yamila Cardoso

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Molecular and taxonomic evidence unmask hidden species diversity in the genus

***Acrobrycon* (Characiformes, Characidae)**

YANINA F. BRIÑOCCOLI^{1,6}, SERGIO BOGAN², DAHIANA ARCILA³, JUAN J. ROSSO^{4,6}, EZEQUIEL MABRAGAÑA^{4,6}, SERGIO M. DELPIANI^{4,6}, JUAN MARTÍN DÍAZ DE ASTARLOA^{4,6}, YAMILA P. CARDOSO^{5,6*}

Revision of genus *Acrobrycon*

Briñoccoli Y.F. *et al.*

¹ Laboratorio de Ictiofisiología y Acuicultura, Instituto Tecnológico Chascomús, Universidad Nacional de San Martín. Av. Intendente Marino km 8200 CC 164 7130, Chascomús, Buenos Aires, Argentina.

² Fundación de Historia Natural “Félix de Azara”, Centro de Ciencias Naturales, Ambientales y Antropológicas, Universidad Maimónides. Hidalgo 775 piso 7, C1405BDB, Buenos Aires, Argentina.

³ Sam Noble Oklahoma Museum of Natural History and Department of Biology, University of Oklahoma, Norman, Oklahoma, 73072, USA.

⁴ Grupo de Biotaxonomía Morfológica y Molecular de Peces, Instituto de Investigaciones Marinas y Costeras (IIMyC), Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata. Funes 3350, 7600 Mar del Plata, Argentina.

⁵ Laboratorio de Sistemática y Biología Evolutiva, Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata. Paseo del Bosque S/N, B1900FWA, La Plata, Buenos Aires, Argentina.

⁶ Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina, Godoy Cruz 2290, CABA, Argentina.

*Corresponding author: yamilapcardoso@gmail.com

ABSTRACT

We conducted a review of the Neotropical genus *Acrobrycon*. A previous study synonymized the two recognized species in the genus: *A. ipanquianus*, distributed from the western portion of the Amazon River to the northwest region of the La Plata River Basin, and *A. tarijae*, described from the Lipeo River in Bolivia. We revisited this result by collecting additional morphometric, meristic, and genetic data (COI mitochondrial gene) for 24 individuals distributed along La Plata River Basin in Argentina, and discussed our results in the context of multiple biogeographic process of isolation in this basin. Our results revealed a more complex history of diversification and geographic distribution across *Acrobrycon* species than previously suspected. We present new evidence that leads us to reconsider the validity of *A. tarijae*, which is distinguished from *A. ipanquianus* by the number of vertebrae (37-39 vs. 41-42), pleural ribs (12-13 vs 14), and unbranched anal-fin rays (v-vi vs. ii-iii). These results were supported by molecular analysis which showed a genetic divergence >4% between both nominal species, also, indicated two major genetic clusters of *A. tarijae*: the first group consisted of specimens from the Bermejo, Pilcomayo, Itiyuro and Juramento River Basins (northern Argentina), and the second group is composed by specimens from the southernmost basins, such as the Salí River in Tucumán and the Quinto River in the province of San Luis. These results suggest that the genetic structure may be a consequence of the geographical distance that separates the two groups and the type of drainage.

Keywords: Freshwater fishes, Stevardiinae, mitochondrial DNA, Endorheic, La Plata River Basin.

INTRODUCTION

In recent decades, the field of phylogenetic systematists have embraced ground-breaking innovations, from sophisticated tools for collecting and archiving samples to improved web databases, images, analytical software and DNA sequencing approaches. Taxonomic research is central in biological studies to ensure the precise species delimitations and identification of the biology units under study. Taxonomy is an arduous and difficult job to carry out, in part due to the lack of specialists, but also to the magnitude of the Earth's biodiversity (Goldstein and DeSalle, 2011).

The family Characidae, with over 1,180 valid species, is the most diverse family of the order Characiformes (Fricke et al. 2021). The phylogenetic relationships within Characidae have been largely controversial based on reproductive, morphological and molecular characters (Javonillo et al. 2010; Mirande, 2010). For instance, the genus *Acrobrycon* Eigenmann and Pearson 1924 was initially proposed as most closely related to the genera *Diapoma* and *Planaltina* based on two reproductive characters, the presence of pheromone organs overlying the basal portions of the caudal fin, comprising the tribe Diapomini (Weitzman et al. 1988). Subsequent morphological studies, examining a large number of osteological and external characters suggested instead that *Acrobrycon* was most closely related to *Mimagoniates*, *Pseudocorynopoma*, and *Diapoma* (Mirande, 2010). More recently, the examination of molecular characters provided additional insights about the relationships of *Acrobrycon*, suggesting a closer association with the genus *Hemibrycon* (Thomaz et al. 2015). As both genera shared with *Boehlkea* the presence of teeth along more than one-half the length of the dentigerous margin of the maxilla, Thomaz et al. (2015) provisionally included these three genera in the reclassified tribe Hemibryconini. Vanegas-Ríos et al. (2018) placed *Acrobrycon* outside of Hemibryconini

and as a sister clade of the Stevardiini tribe. Later, based on a combined phylogenetic analysis Mirande (2019) also showed the sister relationship between *Acrobrycon* and *Hemibrycon*, including both genera within Hemibryconii, a result also shown in Vanegas-Ríos et al. 2020.

In addition to the multiple reassessments of the phylogenetic placement of the genus *Acrobrycon*, this genus has also a complex taxonomic history (see supplementary information). Currently, *Acrobrycon* is composed by three species that were described using only morphological characters (Arcila et al. 2013). *Acrobrycon ipanquianus* (Cope, 1877) is distributed from the western portions of the Amazonas Basin through to the north-western region of the La Plata River Basin, including a synonymic species, *A. tarijae* Fowler 1940. *A. starnesi* Arcila, Vari and Menezes 2013, only known from the Thyumayu River in the southwestern portion of the Amazon Basin in Bolivia, and *A. ortii* Arcila, Vari and Menezes 2013, distributed in the upper Paraguay River in Bolivia.

Here, we generated new molecular and morphological data of multiple specimens of *Acrobrycon* to assess the genetic diversity of the genus and a reassessment of the validity of *A. tarijae* across La Plata River Basin. Genetic diversity is discussed in the context of three biogeographic processes: isolation-by-distance (IBD; Wright, 1943), isolation-by-environment (IBE; Wang and Bradburd 2014), and isolation-by-barrier (IBB; Rahel, 2007).

MATERIALS AND METHODS

Molecular approach

Phylogenetic reconstruction and haplotype network

A total of 24 individuals of *Acrobrycon* from 11 localities of La Plata River Basin in Argentina were included in all molecular analyses (Figure 1). The mitochondrial cytochrome c oxidase I (COI) gene was amplified at the Argentine International Barcode of Life Laboratory reference (IIMyC, CONICET, Mar del Plata, Argentina) and the Canadian Centre for DNA Barcoding, Biodiversity Institute of Ontario (CCDB, University of Guelph, Guelph, Ontario) using the cocktail primers described by Ivanova et al. (2007). Sequencing was performed in the CCDB. A molecular phylogenetic approach was used to describe the genetic relationships between the *Acrobrycon* species and some *Hemibrycon* sequences available in GenBank. The genera *Nantis* and *Cyanocharax* were used as outgroups. Maximum likelihood (ML) analyses were conducted using MEGA 7.0.26 (Kumar et al. 2016). Branch support was assessed using the bootstrap algorithm with 1000 replicates.

The minimum spanning network of the COI haplotypes of the *Acrobrycon* sequences was constructed using PopART 1.7 (Leigh and Bryant 2015) to assess the connections and frequencies of haplotypes. The network was coloured following the Barcode Index Number (BIN) as indicated below. To assess the role of geographical distance on the genetic structure of the genus *Acrobrycon*, we used the Mantel test (Mantel, 1967) with the aim of testing by the presence of an isolation-by-distance process. We estimated a matrix of genetic distances using the Kimura two-parameter (K2P) model (Kimura, 1980) in MEGA, and a matrix of geographical distances based on individual pair comparisons. To estimate the role of isolation-by-barrier, we classified the sampling localities using two schemes: endorheic basin (with no connection to the sea) or exorheic basin (connecting with the sea). The phenomenon of basin fragmentation isolates the aquatic organisms that inhabit these rivers, resulting in an increase of the genetic diversity (Berry

et al. 2019). If the time of isolation is long enough, it can result in population genetic differentiation within the species that has been fragmented. But if the time of isolation is even greater, it may eventually lead to allopatric speciation (Briñoccoli et al. 2021). Finally, to assess whether the isolation-by-environment is structuring the haplotype network of *Acrobrycon* species, we measured the altitude in meters above sea level (MASL) for each site in which specimens of *Acrobrycon* were collected.

Diversity and divergence

Species delimitation analyses were conducted using the Automatic Barcode Gap Discovery (ABGD) method (Puillandre et al. 2012) and the BIN. For the ABGD, the COI alignment was uploaded to the online platform (<http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html>), and run using two schemes: the default settings ($P_{min} = 0.001$, $P_{max} = 0.1$, Steps = 10, X relative gap width = 1.5, Nb bins = 20), and the Kimura distance models. The BIN was generated and downloaded for all 22 sequences available on the BOLD database. The BIN analysis clusters barcode sequences to create Operational Taxonomic Units (OTUs) that closely reflect species groupings. We computed the distance matrix of the K2P model (Kimura, 1980) using MEGA following the groups identified in the ABGD and BIN analyses. Finally, we explored whether the groups identified by the BIN and ABGD analyses were consistently supported by the ML analysis and the morphological characters (Tables 1 and 2).

Taxonomic revision

All the specimens examined are shown in the Supplementary Information. Measurements rounded to the nearest 0.1 mm were made with digital calipers. Counts and measurements were conducted following previously standardized configurations (Fink and Weitzman, 1974; Menezes and Weitzman, 1990, 2009; Menezes et al. 2003). In order to standardize

as much as possible our comparisons with previous studies examining all species in the genus *Acrobrycon*, measurements and counts were directly compared to those obtained by Arcila et al. (2013).

Meristic counts are provided in the species description. Vertebral counts were taken from clarified and stained specimens (CFA-IC-10058) prepared following the proposed procedures by Taylor and Van Dyke (1985) and dried skeletons (CFA-IC-9504 and CFA-IC-9505). Additional X-ray images for the holotypes of *A. tarijae* (Holotype: ANSP 68775, paratypes ANSP 68776, and ANSP 68778) and *A. ipanquianus* (Holotype ANSP 21114 and paratypes ANSP 21115) were obtained to count the number of vertebrates. The number of vertebrae of the Weberian apparatus was quantified as four elements, and the first pre-ural center and the first fused ural of the caudal fin were counted as a single element.

RESULTS

Molecular approach

Phylogenetic reconstruction and haplotype network

A total of 22 sequences from the original 24 specimens of *A. tarijae* processed were obtained and deposited in GenBank (accession numbers: MW940261-MW940282). COI sequences were also uploaded to BOLD under the project “COIPE Peces Argentinos”. Sequences of two specimens of *A. ipanquianus* from the Amazon Basin, Peru and 18 species from the genus *Hemibrycon* were obtained from GenBank for downstream phylogenetic comparisons. The Tamura-Nei model+G+I (TN93+G+I) was chosen as the best nucleotide substitution model under the Bayesian Information Criterion.

Our phylogenetic results supported the sister-group relationships between the genera *Acrobrycon* and *Hemibrycon* with strong bootstrap support (100%; Figure 4A). The

relationships within *Acrobrycon* resulted in two major clades, the first one including only the specimens of *A. ipanquianus*, and the second clade encompassing the 22 specimens of *A. tarijae* from La Plata River Basin.

We found a total of 11 different haplotypes of *A. tarijae* from La Plata Basin in Argentina, and one haplotype in *A. ipaquianus* from Peru (Figure 4B). The haplotype network provided strong support differentiating *A. ipanquianus* from *A. tarijae*. Interestingly, it also identified two haplotype groups across the specimens of *A. tarijae* that differ in their assigned BIN and in their distinctive geographic distribution (Figure 4C). This result is consistent with the inferred phylogenetic tree (Figure 4A), and the Mantel test that showed a significant correlation between geographic and genetic distances among the *A. tarijae* specimens ($p = 0.008$), indicating an isolation-by-distance (IBD) process.

Regarding the type of basin (IBB), the sites located in the north of Argentina (Salta and Jujuy) are part of exorheic basins, while those sampling sites towards the centre-south of the country (Tucumán, Córdoba and San Luis) are part of endorheic-arheic basins (Figure 4A). We also found the two groups of haplotypes separated from each other by eight mutations in the haplotype network coloured according to BIN values (Figure 4B). In terms of environment (IBE), altitudes in sampling sites spanned from 182 to 883 MASL.

Diversity and divergence

Three Barcode Index Numbers (BINs) were obtained for the genus *Acrobrycon*. The ACW0596 barcode was assigned to two specimens of *A. ipaquianus* from Peru. We found two barcodes (ACM2250, ADE4913) associated to *A. tarijae* in Argentina. The barcode ACM2250 was assigned to seven specimens from Salí, Bermejo, Cuarto and Quinto riv-

ers in Argentina, while the barcode ADE4913 was found on nine individuals from Bermejo and Pilcomayo rivers in Argentina. However, the Automatic Barcode Gap Discovery (ABGD) analysis only reported two groups, one corresponding to the ACW0596 BIN and another corresponding to both ACM2250 and ADE4913 BINs together. The within-BIN distances were zero for the ACW0596 and 0.01% for both ACM2250 and for ADE4913. The between-BIN distances were 1.87% ACM2250-ADE4913; 4.2% ACW0596-ACM2250 and 4.1% for ACW0596-ADE4913.

Taxonomic revision

Acrobrycon Eigenmann and Pearson, 1924

Type species.—*Tetragonopterus ipanquianus* Cope, 1877, by original description (Figure S1).

Acrobrycon tarijae Fowler, 1940:50 (Figs 2-S2) [Type locality: Lipeo River, branch of Bermejo River, Department of Tarija, Bolivia (currently the Lipeo River type locality was relocated to the province of Salta, Argentina)]. Tables 1-2.

Amended diagnosis. *Acrobrycon tarijae* is distinguished from *A. ipanquianus* by the lower number of vertebrae (37-39 vs. 41-42), and pleural ribs (12 to 13 pairs vs. 14 pairs in the type series), and the higher number of unbranched anal-fin rays (v-vi vs. ii-iii). *Acrobrycon tarijae* is separated from *A. starnesi* by the number of perforated scales of the lateral line (51 to 60 vs. 61 to 66), and the number of horizontal scale rows around the caudal peduncle (19 to 21 vs. 22 to 26). *Acrobrycon tarijae* can be distinguished from *A. ortii* in the number of branched anal-fin rays (23 to 27 vs. 19 to 21).

Description. The description of *A. tarijae* follows Arcila et al. (2013), including some adjustments to the ranges as indicated below. *Acrobrycon tarijae* is a characid of moderate bearing (can exceed 114 mm of SL), with an elongated body. Greater body

depth in the sector behind the origin of the pectoral fins and before the dorsal-fin origin. Dorsal profile convex from the tip of the snout to the origin of the dorsal fin, slightly depressed along the nape, almost straight along the base of the dorsal fin and slightly concave along the caudal peduncle. Ventral profile convex. Dorsal-fin origin anteriorly than the origin of the anal fin. Rounded muzzle in lateral view. Terminal mouth slightly upwards. Maxilla extending posteriorly beyond the vertical through the anterior margin of the orbit, but not reaching the vertical through the posterior border of the pupil. Posteroventral border of the maxilla convex and posterior margin concave. Dorsal-fin rays ii+8. Some specimens with posteriormost dorsal ray unbranched and others with a branched condition in this ray, although this is restricted to a very small part of its distal tip. Small adipose fin. Anal fin with v,vi-23-26. Pectoral fin with i, 9-10. Pelvic-fin rays i, 6-7. Caudal fin with two well differentiated lobes, with i,9-10.

The tip of the pelvic fin does not reach the anal-fin origin, cycloid scales, with 4 to 8 rays along the exposed surface over most of the body and 14 to 17 rays on scales bordering the opening of the caudal pocket. Lateral line with 51-60 perforated scales. Predorsal scales 19-26. Horizontal scale rows around caudal peduncle 19-21. Premaxillary teeth in two different rows, outside row with 4-5 teeth and internal row with 4 teeth. Larger teeth with five cusps; smaller teeth with three cusps. Maxillary teeth 6-11. Larger anterior maxilla teeth with 1-3 cusps, other smaller teeth with 1-2 cusps. Dentary with 4 large anterior teeth with 5 cusps, followed by 6-10 smaller teeth with 1-3 cusps.

Sexual dimorphism. Mature males present a hypertrophied terminal caudal-fin squamation forming a caudal pocket and also have bony hooks on the anal, pelvic, and caudal -fin rays.

Coloration in live specimens. Gray ocher dorsal coloration from the nostrils to the caudal peduncle. Silvery flanks, paler at ventral section and with purplish-bluish reflection dorsally. A golden to greenish coloration bordering the lateral line. A silver wide band behind the humeral spot progressing distally to the end of the body, continuing with a marked black pigmentation in the medial rays of the caudal fin. Circum-orbital bones generally silver, the fifth and sixth infraorbitals may have purplish reflections. Operculum with conspicuous violet reflections, turning greenish above and ahead this bone. A conspicuous humeral dark spot vertically elongated. Pectoral and ventral fins whitish. The dorsal and adipose fins smoothly yellowish. The anal fin gray or yellowish usually with a whitish lower margin (Figure S2).

In alcohol- preserved specimens, body brown, with darker dorsum. Humeral spot dark, with a well-defined upper part and a fainter lower expansion. In many specimens the lower expansion of humeral spot absent. The lateral band dark, thin at the level of the humeral spot, and shortly wider until the distal end of the body. This band conspicuous in some specimens and very faint in others. The middle rays of the caudal fin black.

DISCUSSION

Species diversity of Acrobrycon

Our study revealed an unexpected genetic diversity for the genus *Acrobrycon* along La Plata River Basin as well as additional informative morphological characters. This integrative approach allows us to revalidate *A. tarijae*, which is distinguished from *A. ipanquianus* by the lower number of vertebrae and pleural ribs, a higher number of unbranched anal-fin rays and by its genetic identity and distance from other valid species of the genus. The genetic analyses showed a clear discrimination between *A. ipanquianus*

and *A. tarijae*. The morphological traits of the specimens of *A. tarijae* showed clear differences with the two additional species described for this genus, *A. ortii* and *A. starnesi* (Arcila et al. 2013). Furthermore, the molecular analyses showed a conspicuous genetic structure within *A. tarijae* revealing the existence of two mitochondrial lineages. The BIN analyses assigned two different OTUs to these lineages which diverged by 1.87%. In fishes, COI sequences have traditionally been used to delimit species and a 2% paired divergence threshold has been proposed to discriminate interspecific from intraspecific genetic divergence (Ward, 2009, 2012). However, the use of only this genetic criterion for taxonomic decision has been controversial (Cardoso et al 2019) In *Hypostomus*, for example, it was shown that 82% of the pairs of sister species that involved at least one well-defined morphological species, showed divergence values of less than 2% (Jardim de Queiroz et al. 2020). Despite our genetic results obtained a distance close to 2% of differentiation, our examination of the morphological characters did not find unequivocal characters for the description of a new species.

Genetic structure within Acrobrycon tarijae

The two BINs assigned for *A. tarijae* presented two well-delineated groups consistent with their geographic distribution. Whereas specimens of BIN ADE4913 were collected from northern drainages, those belonging to the BIN ACM2250 are from the southern and northern drainages of Argentina. Moreover, the Mantel test also supported the presence of two groups within *A. tarijae* isolated by geographic distances. However, the isolation by environment, using the altitude as the discrimination variable (Briñoccoli et al. 2021) and the isolation by barrier, considering the condition of arheic or endorheic of studied basins did not seem to affect the genetic structure of *A. tarijae*.

Our results indicated that the distribution of *A. tarijae* is restricted to the western headwaters of La Plata Basin (Liotta, 2005), including an important sector of the Chaco plain on the main channel of the Pilcomayo River and the dry Chaco sector of the Bermejo basin in Formosa (Ringuelet et al. 1967). The geographic coverage of our samplings extends the distribution of *A. tarijae* to the Quinto River basin in San Luis, being the only record known for the province of San Luis and the southernmost documented report for this species (Cardoso et al. 2015). However, the abundance of this species is relatively low compared to the populations distributed in the Andean foothills or the Pampas mountains, also linked to landscapes of the foothills (Mirande and Aguilera 2009; Monasterio de Gonzo, 2003). *Acrobrycon tarijae* is an abundant species in some endorheic basins as the Itiyuro, the Horcones and the Urueña rivers (Monasterio de Gonzo et al. 2006; Monasterio de Gonzo, 2003), and in the large arheic system of Mar Chiquita Lagoon Basin (Butí and Miquelarena, 1995; Haro and Bistoni, 1996; Haro et al. 1991). These endorheic basins are characterized by high levels of endemic freshwater fishes (Miquelarena and Menni, 1999). However, our results indicated a wide range distribution for *A. tarijae*, even more extensive than the one observed for the catfish *Rhamdella aymarae* (Briñocoli et al. 2018).

Morphological variations in Acrobrycon

The morphological study by Arcila et al. (2013) characterized *A. ipanquianus* (including *A. tarijae* as a synonymous species) as a species with a relatively high body, a trait that would significantly differentiate it from *A. ortii*. However, in the series of types of this species (holotype and more than 33 paratypes) some individuals (see for instance Figure 2 B-D) have a low body with similar proportions similar to those described for *A. ortii*.

According to Arcila et al. (2013) the explanation for the lack of variation within the *Acrobrycon* species may be the relatively homogeneous habitats occupied by these species and their moderate-sized geographic distributions. In fact, two of the three *Acrobrycon* species are only known from type localities. However, in the La Plata River Basin, a great morphological variation of body height was observed in *A. tarijae*. No link was found between observed genetic differences and these morphological variations. In our survey, elongated, low and well-stylized bodies are observed in individuals that inhabit rivers with strong currents, sometimes linked to mountain formations or in the beds of large rivers, such as the Pilcomayo River. Morphological differences between populations of the same species could be due to the environmental conditions to which the fish are exposed. The lack of diagnostic value of body depth to discriminate *Acrobrycon* species is evident. Therefore, to differentiate *A. ortii* from other species, it is important to count the number of branched rays in the anal fin (19-21 vs 23-27 in *A. ipanquianus* and *A. tarijae*). Unfortunately, the molecular identity of *A. ortii* and *A. starnesi* could not be assessed in this study, but these results urge the inclusion of these species along with additional populations of *Acrobrycon* in future studies to investigate their population structure and phylogenetic relationships as well as biogeographic and morphological patterns of diversification.

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DISCLOSURE STATEMENT

No potential conflict of interest was reported by the authors.

SUPPLEMENTAL DATA

Supporting information 1: Remarks on the history description of the genus *Acrobrycon* and its species. Supporting Information 2: List of all Material examined and supplementary figures.

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AUTHOR CONTRIBUTIONS

YFB, SB, YPC: collection samples, data curation, formal analysis, resource, writing-original draft. **JJR, EM, SD, JMDA:** collection samples, data curation, resource, writing-review and editing. **DA:** data curation, resource, writing-review and editing. **SB, YPC:** conceptualisation and project administration.

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TABLES

Table 1. Morphometric measurements of 30 specimens of *Acrobrycon tarijae*. The standard length (SL) is expressed in mm and all other measurements are expressed as a percentage of SL, except for head subunits which are expressed as percentages of the head length.

	Range	Mean±S.D.
Standard length (mm)	450-900	625.83±129.18
percent of SL		
Depth at dorsal-fin origin	118-305	171.83±52.42
Snout to dorsal-fin origin	228-478	334.83±68.20
Snout to pectoral-fin origin	105-209	151.67±27.10

Snout to pelvic-fin origin	206-453	288.13±66.61
Snout to anal-fin origin	264-597	390.57±89.18
Caudal peduncle depth	50-120	77.67±17.38
Caudal peduncle length	44-130	77.53±22.95
Pectoral-fin length	13.1-186	126.64±33
Pelvic-fin length	56-128	91.70±17.65
Dorsal-fin base length	42-110	74.57±20.22
Dorsal-fin height	86-183	131.40±25.71
Anal-fin base length	16.2-276	173.31±48.61
Anal-fin lobe length	128-337	219.07±46.29
Eye to dorsal-fin origin	183-400	269.97±58.10
Dorsal-fin origin to caudal-fin base	185-455	313.90±69.81
Head length	98-192	141.13±27.15
percent of HL		
Horizontal eye diameter	30-57	44.23±6.72
Least interorbital width	30-63	44.70±8.90

Table 2. Meristic data taken in 30 specimens of *A. tarijae*.

Lateral line scales	51-60
Dorsal-fin rays	ii,8
Anal-fin rays	v-vi, 23-27
Pelvic-fin rays	i,6-7
Pectoral-fin rays	i,9-11
Maxillary teeth	6-11
Vertebrae	37-39
Pleural ribs	12-13

FIGURES

Figure 1. Map of the study area for the species of the genus *Acrobrycon*. The stars represent the type localities for each species, the circles the localities with sequenced specimens and the diamonds the localities without sequenced specimens.

Figure 2. Types of *A. tarijae*. A: Holotype; B-E: Paratypes. The scale represents 1 cm.

Figure 3. Count of vertebrae in Holotypes. A: *A. tarijae* and B: *T. ipanquianus*. The abbreviation W represents the first four vertebrae of the Weber complex.

Figure 4. A: Maximum Likelihood tree of *Acrobrycon* based on 521 nucleotides of the mitochondrial gene COI. Bootstrap values are shown above the branches, values below 70 are not shown. In the specimens under study of *A. tarijae*, the access number to the Genbank is found and the asterisk indicates those that were sampled in endorheic basins. B: Haplotype network, coloured by three BINs groups. C: Map with the sampling sites of the *A. tarijae* specimens.







